ACS Sustainable Chemistry and Engineering, Volume 9, Issue 30, August 2021, pp.10381-10391
DOI: 10.1021/acssuschemeng.1c03951

Integrated Fermentative Production and Downstream processing of 2,3-Butanediol from Sugarcane bagasse-derived Xylose by Mutant Strain of *Enterobacter ludwigii*

Yassin Amraoui [a*], Vivek Narisetty [a*], Frederic Coulon [a], Deepti Agrawal [b], Anuj Kumar Chandel [c], Sofia Maina [d], Apostolis Koutinas [d], Vinod Kumar [a]**

[a] School of Water, Energy and Environment, Cranfield University, Cranfield MK43 0AL, UK

[b] Biochemistry and Biotechnology Area, Material Resource Efficiency Division, CSIR-Indian Institute of Petroleum, Mohkampur, Dehradun 248005, India

[c] Department of Biotechnology, Engineering School of Lorena (EEL), University of São Paulo, Lorena- 12.602.810, Brazil

[d] Department of Food Science and Human Nutrition, Agricultural University of Athens, Iera Odos 75, Athens, Greece

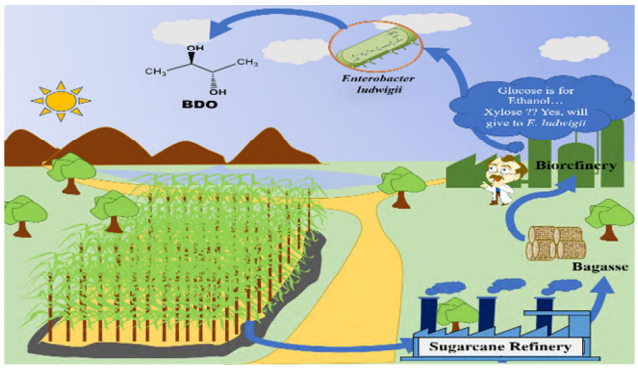
* Contributed equally

**Corresponding author

Phone: +44(0)1234754786

E-mail: Vinod.Kumar@cranfield.ac.uk

Graphical abstract



Sugarcane bagasse, the residue from sugarcane processing industries, can be saccharified to readily available renewable sugars for the sustainable production of 2,3-butanediol.

Abstract: In this study, a mutant strain of *Enterobacter ludwigii*, developed in our previous work, was evaluated to utilize pure xylose as the sole carbon and energy source for 2,3-butanediol (BDO) production. Later, this strain was also investigated on detoxified and non-detoxified xylose-rich hydrolysate obtained from hydrothermally pretreated sugarcane bagasse (SCB) for BDO production. Supplementing the fermentation medium with 0.2% w/v yeast extract improved cell growth (31%), BDO titer (43%) and yield (41%) against the synthetic medium devoid of any complex nitrogen source. The fed-batch culture with cyclic control of pH resulted in BDO production of 71.1 g/L from pure xylose with an overall yield and productivity of 0.40 g/g and 0.94 g/L. h, respectively. The BDO titer, yield and productivity achieved with detoxified hydrolysate were 63.5 g/L, 0.36 g/g and 0.84 g/L.h, respectively. In contrast, 32.7 g/L BDO was produced from non-detoxified hydrolysate with a conversion yield of 0.33 g/g and productivity of 0.43 g/L.h. BDO accumulated on pure xylose and detoxified SCB hydrolysate was separated by aqueous two-phase system (ATPS) method using $(NH_4)_2SO_4$ as salting-out agent and isopropanol as an extractant, resulting in the BDO recovery of more than 85%. The results achieved in the current work exemplify a step towards industrial BDO production from cost-effective hemicellulosic hydrolysates by *E. ludwigii*.

Keywords: Xylose; Detoxified hydrolysate; Non-detoxified hydrolysate; *Enterobacter ludwigii*; 2,3-Butanediol; Aqueous two-phase system (ATPS) extraction

Introduction

Glucose and xylose are the two most abundant sugars in lignocellulosic biomass (LCB), geographically ubiquitous and renewable resource on the earth. LCB, popularly known as second-generation feedstock consists of 30-50% (w/w) cellulose, and 25-35% (w/w) hemicellulose, which upon depolymerization generates glucose and a mixture of sugars containing ~ 90% xylose respectively.[1,2] Second-generation biorefineries currently focus only on cellulosic valorisation to fuels and chemicals, while hemicellulosic and lignin fractions are used for biogas and heat/electricity generation. The net profitability of LCB-based biorefineries could be ameliorated through high-grade applications of hemicellulose/lignin fractions. The transformation of these fractions into high-value products will positively impact the commercial viability of biorefineries and environmental issues such as waste reduction, pollution, and greenhouse gas emissions. Like glucose, xylose is also a fermentable sugar and can be transformed into a spectrum of products, e.g. organic acids, alcohol, polymers, lipids, etc.[3,4] The combined use of glucose and xylose can increase the utilization ratio of LCB by more than 50%.[5,6] The biorefineries often overlook the application of xylose as a potential feedstock, owing to the lack of efficient xylose metabolizing microbial cell factories. This problem is exacerbated by the glucose repression effect, preventing xylose assimilation in the presence of glucose. Thus, there lies a tremendous scope to convert this challenge into an opportunity and exploit the potential of hemicellulose/xylose as valuable feedstock. In the last decade, significant efforts have been focused on valorising hemicellulosic streams into several valuable products via a biorefining approach.[4]

2,3-Butanediol (BDO) is an industrial chemical with multiple applications in the food, cosmetics, and pharmaceutical industries. BDO can also be transformed into other speciality and value-added chemicals. For example, BDO can be dehydrated to like methyl ethyl ketone (MEK) and 1,3-butadiene (1,3-BD); MEK is an industrial solvent, and 1,3-BD is a precursor for synthetic rubber. Due to the high heat of combustion (27.2 kJ/g), BDO can be a fuel additive for aviation fuels. It has been estimated that the global market for BDO derivatives itself is~32 million tons per annum, valued at $43 billion.[7 - 9] Currently, the commercial demand of BDO is met through the petrochemical route. However, the LCB-based biochemical platform, being green and sustainable, can provide a competitive edge besides being economical. The fermentative route for BDO production involves condensation of two moles of pyruvate to yield α-acetolactate followed by decarboxylation to acetoin or diacetyl, which upon reduction produce

BDO. The enzymes involved in the pathway are α-acetolactate synthase, α-acetolactate decarboxylase, diacetyl reductase and butanediol dehydrogenase (Supplementary Figure 1).[7,9]

In the current work, we demonstrated BDO production from xylose by a mutant strain of *Enterobacter ludwigii*, developed in our previous work.[10] The novelty of the work was to evaluate the robustness of the said mutant in producing and purifying BDO using crude xylose derived from sugar cane bagasse (SCB) and compare product titres and productivity against pure xylose. Initially, the mutant strain was evaluated on pure xylose, as a sole carbon source. Later, its efficacy to assimilate xylose from non-detoxified and detoxified SCB hydrolysate obtained from hydrothermal pretreatment was assessed. The impact of initial xylose concentration, nitrogen sources, modes of cultivation and pH were examined on xylose uptake, biomass and BDO formation. Finally, BDO accumulated on pure xylose and detoxified SCB hydrolysate was extracted by aqueous two-phase system (ATPS) method using $(NH_4)_2SO_4$ as salting-out agent and isopropanol as extractant.

Materials and Methods

Materials

All the chemicals used in this study were purchased from Sigma Aldrich (USA) and Fischer Scientific and were of analytical grade. The non-detoxified xylose-rich hydrolysate obtained after hydrothermal pretreatment of SCB was kindly provided by our industrial partner Nova Pangaea Technologies (https://www.novapangaea.com), Redcar, UK. The hemicellulosic hydrolysate composition was as follows (g/L): 18.5, xylose; 1.2, glucose; 1.5, arabinose; 1.0, galactose; and 2.2, acetic acid. The detoxification was performed by our other industrial partner, Green Fuels Research (http://www.greenfuels.co.uk). Over liming and activated charcoal detoxified the hydrolysate, with the complete elimination of acetic acid and furfural. After detoxification, the hydrolysate contained (g/L): 14.0, xylose; 3.5, glucose; 2.0, arabinose; 1.5, galactose. Around 5 litres of the hydrolysate was subjected to rotary vacuum evaporation (Rotavapor, BUCHI UK Ltd). The set-up was maintained at 100 mbar and 80˚C, overnight to obtain a concentrated xylose rich SCB hydrolysate with a total sugar concentration of 400 g/L. Both the non-detoxified and detoxified xylose rich hydrolysates were diluted appropriately for media preparation, depending on the experimental requirements.

Microorganism and culture conditions

The bacterial strain *Enterobacter ludwigii* has been isolated at the Laboratory of Food Microbiology and Biotechnology at the Agricultural University of Athens, Greece.[11] The current study made use of mutant strain of *E. ludwigii*, constructed in our previous study.[10] The submerged cultivations were carried out using synthetic medium [12] in 500 mL shake flasks containing 100 mL working volume. The medium was supplemented with pure xylose or xylose rich SCB hydrolysate to the desired concentrations. The final pH of the medium before sterilization was adjusted to 6.6 using 5 M NaOH. The flasks were inoculated

with 2% (v/v) of fresh inoculum and incubated at 30 °C under constant shaking at 180 RPM on a rotary shaker (Excella 24, New Brunswick, USA). During these experiments, the pH stat conditions were not maintained. However, routinely samples were withdrawn and checked for pH variation during the fermentation. Unless otherwise stated, all of the shake flask experiments conducted in this study were carried out under similar conditions.

Effect of different initial levels of pure xylose, non-detoxified and detoxified xylose rich hydrolysate

The effect of initial concentration of pure xylose (20 – 100 g/L), non-detoxified and detoxified xylose hydrolysate (20 – 60 g/L) on substrate uptake, cell growth and BDO production was investigated in shake flask.

Effects of different nitrogen sources

The synthetic medium is devoid of any organic/complex nitrogen source. The effect of additional fortification with organic nitrogen sources (urea, corn steep liquor and yeast extract) at 2 g/L respectively, on xylose (pure) consumption, cell growth, and BDO production was investigated using the synthetic medium mentioned above and the results were compared with control cultures in shake flask.

Bioreactor studies

The batch experiments were performed in a 2.5 L benchtop bioreactor (Electrolab Bioreactors, UK) with a 1.0 L working volume and an inoculum size of 10% (v/v). The temperature, agitation speed, and aeration rate were controlled at 30 ºC, 180 RPM, and 1.0 vvm, respectively, while pH was not maintained. For fed-batch fermentation, the initial xylose levels and pH were 40 g/L and 7.0, respectively. The pH was controlled in a cyclic manner. Once the pH of the medium dropped below 5.0, it was brought back to 7.0 by the addition of 5 N NaOH. Three different runs were performed with pure xylose, non-detoxified and detoxified xylose rich hydrolysate, both in batch and fed-batch mode. The initial xylose concentration in batch culture was 60 g/L, while the fed batch started with 40 g/L. The culture was fed intermittently with a concentrated solution of pure xylose and hydrolysate containing 350-400 g/L xylose to maintain residual xylose at or above 20 g/L.

Aqueous two-phase system (ATPS) extraction of BDO from fermented broth

The fermented broth from fed-batch culture of *E. ludwigii* on pure xylose and detoxified hydrolysate in bioreactor was centrifuged at 8000 rpm for 10 mins to remove the microbial cells and other insoluble macromolecules. The clear fermentation broth obtained was used for recovery of BDO using ATPS extraction with $(NH_4)_2SO_4$ and isopropanol as salting-out agent and extractant, respectively.[13] Commercial grade $(NH_4)_2SO_4$ was added to supernatant with a final salt concentration of 20% w/v. The mixture was vortexed for 15 minutes followed by addition of isopropanol with equal volume of fermented broth (1:1). The mixture was vortexed again for 15 minutes and put in a stationary mode for 7-8 h at room

temperature for phase separation to occur. The BDO was concentrated from top organic phase through vacuum distillation (Rotavapor, BUCHI UK Ltd) operating at 45 ^{0}C, 150 mbar pressure. The concentrated BDO obtained was checked for BDO and presence of other metabolites as mentioned below. The parameters partition co-efficient (K) and recovery yield (Y) which reflects the efficacy of the process were computed using following equations.

$$K = \frac{C_T}{C_B} \quad \text{..... (1)}$$

$$Y = \frac{C_T}{C_{FB}} X\ 100\% \text{..... (2)}$$

Where C_T, C_B, and C_{FB}, are the concentrations of BDO in top organic, bottom aqueous phase, fermented broth, respectively.

Analytical methods

The samples were withdrawn periodically and analyzed for cell growth, pH, residual xylose, BDO, acetoin, ethanol, lactic, succinic and acetic acid concentration. The bacterial growth through was quantified by measuring optical density at 600nm. Since a small amount of glucose in hydrolysate was quickly consumed, we could not detect it in the fermentation broth for most of the time points during cultivation. Intermittent pH monitoring during the shake flask experiments was done using a pH meter (pH 100, VWR International, UK). The concentrations of metabolites in supernatant were measured by HPLC (Agilent Technologies 1200 series, USA) and eluted using the Rezex ROA-Organic Acid H+ (Phenomenex, USA) column at 60°C attached with a refractive index detector (RID). The mobile phase and flow rate were 5 mM H_2SO_4 and 0.4 mL/min, respectively. All measurements were conducted in triplicates and the values were averaged. The standard deviation was not more than 10 %.

Results

Shake flask cultivation of *E. ludwigii* at different concentrations of pure xylose

The shake flask studies were conducted to understand the xylose consumption pattern of *Enterobacter ludwigii* and metabolites produced from xylose as sole carbon source. Further, varying initial concentration of xylose from 20-100 g/L, gave a fair idea on the maximum tolerance limit of mutant strain for this sugar. The time course profiles for xylose uptake, cell growth, BDO production and pH are shown in Figure 1. Complete xylose (20 g/L) was exhausted within 20 h concomitant with cell growth. The highest cell concentration (OD_{600}) of 10.8 and BDO production of 7.6 g/L with the conversion yield of 0.41 g/g was obtained at 17 and 19 h, respectively. Since for industrial product titres, high substrate concentrations are always desirable, the robustness of the strain was evaluated at different levels of xylose: 40, 60, 80 and 100 g/L. However, an increase in xylose concentration >20 g/L slowed down the substrate assimilation; xylose not being wholly consumed even after 40 h of cultivation and the effect was more

pronounced at higher concentrations. As a result, the amount of residual xylose in the media enhanced with an increase in initial xylose concentration. The most severe inhibition was observed above 60 g/L xylose concentration. At 60, 80 and 100 g/L initial xylose levels, about ~50% substrate was left unconsumed at 40 h. The mutant exhibited a long lag phase with reduced biomass, BDO yield, and productivity. The maximum cell OD_{600} and BDO obtained at 40-100 g/L xylose were in the range of 9-13 and 7-12 g/L, respectively. There was a marginal increment in $OD_{600,}$ but a significant jump in BDO level was obtained at 40 and 60 g/L xylose with a diminishing yield. Qualitative detection of mixed acids (succinic acid, acetic acid and lactic acid) in HPLC chromatogram strongly corroborated with the visual drop in pH reduction during the course of fermentation. Intermittent monitoring studies revealed that pH of the medium reduced < 5.0 within 20-24 h when initial xylose concentrations were between 20 – 60 g/L. However, this trend was delayed when the xylose concentrations were >60g/L.

BDO production from xylose rich SCB derived hydrolysate

The xylose rich hydrolysate obtained after hydrothermal pretreatment often contains inhibitors such as formic acid, acetic acid, furfural, 5-hydroxymethylfurfural etc which impede microbial fermentations. Consequently, either the use of robust microorganisms or detoxification of hydrolysate is required for effective hydrolysate fermentation.[14] In this context, two independent experiments for BDO production from detoxified (Figure 2) and non-detoxified (Figure 3) xylose rich SCB derived hydrolysate were attempted. This kinetic study with pure xylose clearly indicated that an initial xylose of 60 g/L was the maximum threshold concentration beyond which the mutant exhibited sugar inhibition. With this maximum xylose concentration, BDO production studies were evaluated with xylose-rich hydrolysates before and after detoxification in shake flask with three different xylose concentrations: 20, 40 and 60 g/L. The results obtained with detoxified hydrolysate was similar to pure xylose, as depicted in Figure 2. The xylose consumption at 20, 40 and 60 g/L was 100, 73 and 57%, with maximal BDO titer being 8.3, 9.4 and 10.8 g/L, respectively. Unlike detoxified xylose-rich hydrolysate, the fermentation time with non-detoxified hydrolysate was longer in comparison to pure xylose. In non-detoxified medium, an initial xylose of 20 g/L was fully utilized within 28-30 h along with cell OD_{600} of 8.5 and BDO accumulation of ~6.8 g/L (Figure 3). However, at 40 g/L, ~62% of xylose was assimilated resulting in OD_{600} and BDO titre of 10.1 and 8.5 g/L, respectively. Further, at an initial xylose concentration of 60 g/L the mutant exhibited a more prolonged lag phase with merely 43% xylose utilization. The cell OD_{600} and BDO concentration were reduced to 4.7 and 6.3 g/L, respectively.

Effect of additional nitrogen source on BDO production from xylose

The synthetic medium is devoid of any organic/complex nitrogen sources which have shown stimulatory effect on the accumulation of biomass and BDO.[6,7] Taking this precedence, the impact of three different nitrogen sources [urea, corn steep liquor (CSL), and yeast extract (YE)] was examined for cell growth

and BDO biosynthesis. Each one of them was supplemented at 2 g/L in flask cultures with synthetic medium containing xylose as carbon source. Figure 4 shows batch fermentation kinetics with time course data of xylose consumed, OD_{600}, BDO accumulation and pH. The addition of urea had a marginal impact on cell growth, while the BDO production was unaffected. However, CSL and YE significantly expedited the growth resulting in high cell mass which in turn improved xylose consumption and BDO production (Figure 4). The presence of CSL positively affected biomass and BDO synthesis. The OD and BDO titer improved nearly by 13-15% in comparison to control. The maximum improvement was recorded with YE. The cells in YE rich medium multiplied rapidly with 31% increment in OD_{600} against control (13.4 (Figure 4A) vs. 17.6 (Figure 4D). BDO accumulation was ~10 g/L with 43% enhancement compared to control, and yield (0.48 g/g) obtained was close to maximum theoretical yield (0.52 g/g). These results confirmed that addition of YE, a complex nitrogen source containing various amino acids, and vitamins significantly expedited the cell growth resulting in high cell mass which in turn improved xylose consumption and BDO production Hence, a synthetic medium containing YE at 2.0 g/L was used in further experiments.

Batch cultivation in bioreactor

Once, the best organic nitrogen source was shortlisted, the bioreactor studies were conducted with three different types of xylose streams (pure, non-detoxified and detoxified) wherein the oxygen requirements were met via continuous aeration at 1.0 vvm. Figure 5A, 5B and 5C represent xylose consumption, cell growth (OD_{600}), BDO production and pH variation during batch cultivation in bioreactor with pure xylose, detoxified, and non-detoxified xylose rich hydrolysate, respectively. The substrate uptake rate and cell growth was faster in comparison to the flask culture. The xylose was rapidly metabolized after the onset of the fermentation and during batch cultivation with pure xylose, sugar consumption was accompanied by rapid cell growth and an OD_{600} in the range of 15-18 was recorded within 20-24 h. The BDO production increased with rapid growth, and high cell mass peaked BDO production to 17.8 g/L within 26 h only and a yield of 0.44 g/g was achieved (Figure 5A). Thereafter, xylose was completely depleted, and pH reduced < 5.5. The cells also entered the stationary phase, followed by a slight reduction in cell growth and BDO accumulation. Similar results were obtained when pure xylose was replaced with detoxified hydrolysate. More than 90% xylose was exhausted within 30 h, yielding cell OD_{600} of 17. The highest BDO titer obtained was 18.2 g/L, slightly higher than pure xylose, with yield of 0.43 g/g (Figure 5B). The cells took a longer time for adaptation when the mutant was switched to medium containing non-detoxified xylose-rich hydrolysate. In the first 24h, only 50% xylose was assimilated and thereafter, the uptake rate was picked up (Figure 5C). The maximum cell growth (OD_{600}: 15.2), BDO titer (14.6 g/L) and yield (0.36 g/g) achieved with non-detoxified hydrolysate were substantially lower against pure xylose and detoxified hydrolysate. The fermentation broth analysis indicated acetoin, ethanol, succinic (SA), lactic (LA), and acetic acid (AA) as main by-products. All these metabolites were produced in low concentrations (< 2.0

g/L) except acetic acid during fermentation with non-detoxified hydrolysate. The initial concentration of acetic acid in the non-detoxified hydrolysate was 4-5 g/L, which enhanced ~ two-fold by the end of the fermentation. Since the pH was not controlled during the fermentation a continuous drop in pH was noticed till it reached a range of 5.0-5.5.

Fed-batch cultivation in bioreactor

Fed-batch culture is widely applied in the bioprocessing industry. It can avoid the substrate inhibition by maintaining substrate concentration below the toxic level.[15] In the present study, with intent to maximise the BDO productivity and overcome the bottleneck of substrate inhibition, fed-batch cultivation with the mutant was conducted using pure xylose, non-detoxified and detoxified xylose rich hydrolysates. The initial xylose level was 40 g/L, and upon depletion, culture was fed intermittently to maintain xylose above 20 g/L. In the earlier sections, the shake flask and bioreactor studies affirmed that pH reduction owing to mixed acid production was the key driving force that impeded the mutant's fermentative performance, indicating pH control is vital for smooth BDO production. However, in our previous study, we found that continuous exposure to pH drop through forced pH fluctuations triggers the cells to overproduce BDO (Amraoui et al. 2021). Therefore, unlike batch cultivation, where pH was not controlled, fed-batch culture was carried out with cyclic control of pH. The pH was allowed to drop to a level where it induced BDO production without disrupting cell metabolism and then was restored back. The initial pH of the medium was 7.0, was allowed to drop until it reached 5.0 and restored to 7.0 using 5 M NaOH. Figure 6 shows the time course profiles of xylose consumption, cell growth, pH and BDO accumulation. Like shake flask culture, the results obtained with pure xylose and detoxified hydrolysate were quite similar (Figure 6A & 6B). The lag phase (0-4 h) was shorter, and the initial 30 h was a period of active biomass production; thereafter, it almost levelled off. The highest OD_{600} achieved (23-26) was significantly higher than flask culture but similar to batch cultivation in a bioreactor. BDO production was noticed from 6^{th} h onwards, and culture continuously accumulated it till the end of fermentation, with a dominant production period between 24-56 h. With pure xylose, the final BDO titer was 71.1 g/L, and its overall yield and productivity were 0.40 g/g and 0.94 g/L. h, respectively (Figure 6A, 7A, and 7B). On the contrary, with detoxified hydrolysate, the BDO titer, yield and productivity were 63.5 g/L, 0.36 g/g and 0.84 g/L. h, respectively (Figure 6B, 7A, and 7B). However, the mutant displayed lower performance in the non-detoxified hydrolysate. In this case, the xylose consumption was slow and significantly lower than pure xylose and detoxified hydrolysate. The slow xylose uptake was also reflected in terms of cell growth and BDO synthesis. There was a long lag phase of 14 h followed by a log phase, which continued till 48^{th} h with a maximum OD_{600} of 19.3; thereafter, cell growth declined. The BDO production begun at 10 h onwards followed by a slow and steady increase, ending in accumulation of 32.7 g/L BDO with the lower yield (0.33 g/g) and productivity (0.43 g/L. h) (Figure 6C, 7A, and 7B). The spectrum of by-products (acetoin,

ethanol, succinic, lactic and acetic acid) produced during fed-batch fermentation was the same as batch bioreactor cultivation.

Aqueous two-phase extraction of BDO from fermented broth

The high boiling point of BDO along with high affinity for water complicates its downstream processing. Liquid-liquid extraction has several advantages but suffers from low yields of BDO which can be overcome if augmented with salting out. Salting out is an efficient method for recovery of a large amount of BDO (>80%) from fermentation broth along with removal of most of soluble proteins and organic acids in single step (Dai et al. 2014; 2015; Priya et al. 2021). In the current study, salting out for extraction for BDO from fermentation broth was done using $(NH_4)_2SO_4$ with isopropanol as solvent via ATPS method. The composition of fermentation broth used for this purpose was as follows (g/L): [70.2, BDO; 7.45, acetoin; 3.6, SA; 1.8, LA; 6.99, AA; 2.0, ethanol] on pure xylose and [62.8, BDO; 11.9, acetoin; 7.4, SA; 4.58, LA; 3.16, AA; 3.58, ethanol] on detoxified SCB hydrolysate. The salting out effect caused partitioning of BDO and acetoin from aqueous to organic while in case of organic acids the distribution was much less as major fraction of them was present in ionised/salt form and could not move to top phase. The partition coefficient and recovery yields of BDO and acetoin from the fermented broth are shown in Table 1. The partition coefficient and recovery yield for BDO and acetoin accumulated on pure xylose was 7.33, 7.37, 87.99 and 88.05, respectively. Comparable results were achieved in case of detoxified hydrolysate: K_{BDO} – 6.62, $K_{Acetoin}$ – 7.11, Y_{BDO} – 86.87 and $Y_{Acetoin}$ – 87.68. The pure xylose derived BDO rich liquid obtained after vacuum distillation of organic phase contained 61.77 g/L BDO and 6.56 g/L acetoin while in case of detoxified hydrolysate, BDO and acetoin concentration were 54.6 and 10.25 g/L, respectively. In both the cases other metabolites were found to be in at low levels (<1 g/L). Further improvement in recovery can be brought through optimization of type and content of salt and solvent.

Discussion

In the current work, we have demonstrated BDO accumulation using pure xylose and xylose-rich hydrolysate obtained from SCB. The choice of SCB as starting feedstock was based on its abundant availability globally, accounting for ~540 million metric tonnes, as reviewed earlier. [16, 17] There are isolated reports where xylose has been used as the sole carbon source for BDO production, and with xylose-rich hydrolysates, the case studies are rare (Table 1). To the best of our knowledge, there is no report using xylose rich hydrolysate from SCB for biomanufacturing of BDO. Further, we employed a mutant strain of *E. ludwigii*, a promising but less explored biocatalyst for the biosynthesis of BDO.

Shake flask studies indicated that fermentation proceeded rapidly to completion at low xylose levels, while fermentation with higher xylose concentrations was less efficient. The BDO yields of 7.6 g/L from pure xylose (20 g/L) with a conversion yield of 0.41 g/g with the mutant strain of *E. ludwigii* were

relatively higher than Liakou et al. (2018). They cultured *E. ludwigii* on xylose being the sole carbon source and achieved BDO titer and yield of 6.3 g/L and 0.38 g/g, respectively.[18] We found that at higher concentrations, the amount of residual xylose increased continuously and accordingly, cell growth and BDO accumulation were negatively impacted (Figure 1). A similar pattern was observed with detoxified hydrolysate as well. In contrast, the presence of inhibitors (furfurals, acetic acid and hydroxymethyl furfural) in the case of non-detoxified hydrolysate pronounced inhibitory effect. Substrate inhibition is a well-known phenomenon and usually occurs at concentrations above 50 g/L. This has also been encountered during BDO fermentations. For example, Yu and Saddler (1983) reported that xylose concentration above 50 g/L inhibited BDO production and sugar utilization by *Klebsiella pneumonia*.[19] In our previous work, we also found a similar trend during the biosynthesis of BDO using glucose from the brewer spent grain by the mutant *E. ludwigii* strain, but the initial substrate concentration threshold appeared to be beyond 60 g/L of glucose level.[10] However, in the current study, an initial concentration ≥40 g/L xylose triggered substrate inhibition. These results indicate the sensitivity of strain towards the high substrate and its variable response when the carbon source was changed. Besides substrate inhibition, continuous reduction in pH during fermentation was another issue. A significant drop in pH below 5.0 was observed in all the experiments after 20-24 h of cultivation. The release of mixed acids often accompanies bacterial BDO production. However, the bacterial metabolism is quite sensitive to the pH of the growth medium. The pH drop below a threshold value significantly affects the performance of the key enzymes involved in the biosynthetic pathway of BDO.[15,20] Psaki et al. (2019) investigated the effect of increasing initial molasses concentration from 40 to 200 g/L on cell growth and BDO production by *E. ludwigii* in a shake flask where pH was manually adjusted, unlike our study.[21] They observed inhibitory effects above 160 g/L with a decrease in specific growth rate and BDO titer, yield and productivity. Therefore, we believe that in the present study, the cumulative effect of the drop in pH and substrate inhibition could be responsible for retarded performance at xylose levels beyond 20 g/L. Organic nitrogen sources are beneficial for the accumulation of biomass and BDO. In current study, we also observed that fortification with CSL and YE was beneficial for cell growth and BDO accumulation (Figure 4). Similar results have been reported by others that augmentation of YE and CSL to growth medium enhances the BDO accumulation.[6, 22, 23] For example, Häßler et al. (2012) investigated impact of YE on BDO accumulation by *Paenibacillus polymyxa* and best results of 111 g/L BDO production were obtained in fermentation medium containing 60 g/L YE.[22] Cho et al. (2015) noticed an improvement of 1.4- and 1.6-fold in cell biomass and BDO production, respectively, by *Klebsiella oxytoca* when culture medium was supplemented with yeast extract (5 g/L) and casamino acid (10 g/L).[23] Similarly, Wang et al. (2016) observed an increment of 31.5% and 51.3% in xylose assimilation and BDO accumulation when YE level was enhanced from 0 to 50 g/L.[6]

BDO is a neutral metabolite and counter measures against acidification. In the absence of external pH maintenance, BDO production is an example of an adaptive mechanism for pH self-control in response to spontaneous pH drop during fermentation.[24] Petrov and Petrova (2009; 2010) exploited this feature associated with microbial BDO synthesis and have reported that forced pH fluctuations by consecutive elevations with definite ΔpH value resulted in notable improvement in BDO production.[25, 26] Similar observations were also made by Yang et al. (2011) that pH fluctuations are favourable for enhancing BDO levels.[15] High BDO production could be attained using an effective fermentation strategy. In the current study, we performed fed-batch cultivation with control of pH in a cyclic manner which eliminated the drawbacks of substrate inhibition and drop in pH, boosting the BDO accumulation. The pH was allowed to drop to a level where it induces BDO manufacturing and at the same does not disrupt cell metabolism. The BDO accumulated using this strategy with pure xylose was 71.1 g/L with a yield and productivity of 0.40 g/g and 0.94 g/L. h. Comparable results were achieved with detoxified hydrolysate: 63.5 g/L, 0.36 g/g, 0.84 g/L. h while the significant drop was obtained with non-detoxified hydrolysate: 32.7 g/L, 0.33 g/g, 0.43 g/L. h (Figure 6 & 7). The detoxification resulted in an improvement of ~2.0-fold in BDO titer and productivity. The difference in the performance of detoxified and non-detoxified hydrolysate could be attributed to the presence of inhibitors in biomass hydrolysate. The increase in the amount of furfural and acetic acid accumulated with feeding of non-detoxified hydrolysate may be the main reason, that reduced sugar uptake and decreased BDO titer and productivity. Furfurals have been found to exert the strongest inhibitory effect on BDO production.[27] Similarly, organic acid toxicity is well-known, and several fermentation processes are inhibited by accumulation of acetic acid in growth medium. The toxic effects of acetic acid start becoming visible at concentrations as low as 5 g/L. The toxicity of acetic acid is two-fold due to pH-based growth inhibition and the negative impact of acetate ions on metabolism.[28] However, contradictory evidence has been provided with *Bacillus vallismortis,* where the acetate concentration below 10 g/L had an activating effect on BDO production.[29] Our results are in partial agreement with Cheng et al. (2010), who investigated BDO production from pure xylose and detoxified corncob hydrolysate by *Klebsiella oxytoca*.[30] They found similar titer and yield of BDO with pure xylose (28.7 g/L, 0.48 g/g) and hydrolysate (25.7 g/L, 0.49 g/g) during batch cultivation at controlled pH. However, BDO productivity was lower in hydrolysate (0.54 g/L. h) than in pure xylose (1.03 g/L. h), indicating slow fermentation with hydrolysate. The fed-batch fermentation with hydrolysate resulted in a maximal 35.7 g/L of BDO with a yield and productivity of 0.50 g/g sugar and 0.59 g/L. h. Acetoin, ethanol, succinic, lactic, and acetic acid were obtained as main byproducts during batch and fed-batch bioreactor cultivation on pure xylose, detoxified and non-detoxified hydrolysates. The simultaneous production and consumption of these metabolites was noticed which is in congruence with the previous reports [13,20,24], and net accumulation at any point of time was not more than 7 g/L (Supplementary Figure 2). Acetic acid

was the most abundant by-product after BDO with all three feedstocks. The situation was exacerbated in non-detoxified hydrolysate, where it was present in substantial amounts from the beginning of the fermentation.

Table 2 compares BDO production from pure xylose and xylose-rich hydrolysate using various strains. Efficient BDO producers include native *K. pneumonia*, *K. oxytoca*, *Enterobacter cloacae,* engineered *Saccharomyces cerevisiae,* and recently isolated *Bacillus vallismortis* strains. A large variation exists in terms of BDO titer, yield and productivity which were in the range of 5 - 85 g/L, 0.20 – 0.52 g/g and 0.18 - 1.35 g/L. h, respectively. The highest BDO titer of 81.4 g/L using xylose solution from corncob hydrolysate and corn liquor (20 g/L) has been reported by *E. cloacae* with a conversion yield of 0.39 g/g and a productivity of 0.72 g/L. h.[31] Unlike our work where forced pH fluctuation was employed, the pH was controlled in case of other bacterial strains. Owing to high pH tolerance, the BDO production study with *S. cerevisiae* was conducted without pH control. The forced pH fluctuation and its maintenance in regular intervals decrease amount of acid or base required during the process. Most of the studies in Table 2 have used a large amount of complex nitrogen sources to accumulate BDO that not only makes the process expensive but also complicates the downstream processing. For example, Xiao-Xiong et al. (2016) obtained 42.7 g/L BDO from xylose with optimal YE level of 35.2 g/L by *K. pneumoniae* after 48 h of batch fermentation.[32] Similarly, Kim et al. (2017) amassed 69.2 g/L BDO using xylose from fed-batch culture of recombinant *S. cerevisiae* with a yield of 0.38 g/g in 244 h. [33] The fermentation medium used for this purpose contained 10 g/L YE and 20 g/L Bactopeptone. On the other hand, BDO levels (63-72 g/L) achieved in present study are comparable or even better than available literature data with just 2 g/L YE using pure as well as crude xylose, making it competitive.

It is difficult to envisage a commercially viable biological production of BDO without taking into account the product recovery because downstream processing contributes to 50-70% of total production cost and is the major bottleneck impeding the process to be industrialized.[34,35] However, there are few reports on removal of BDO from fermentation broth. In this work, we have employed ATPS method for BDO extraction using $(NH_4)_2SO_4$ as electrolyte and isopropanol as the extractant. The extraction resulted in a liquid rich in BDO with little amount of acetoin and traces of organic acids. The partition coefficient and recovery obtained for BDO and acetoin in current work were similar to Sun et al. (2009). They employed 34% (w/v) isopropanol and 20% (w/v) $(NH_4)_2SO_4$ for extraction of BDO manufactured using glucose as carbon source and *Klebsiella pneumoniae* as biocatalyst. The partition coefficients and recovery yield obtained for BDO and acetoin were 8.29, 8.98, 91.4% and 91.6%, respectively. The distribution of acetoin along with BDO to top phase is a concern but they could be separated using distillation due to significant difference in their boiling points. Since the ATPS method make use of large amounts of salt and solvent, it is essential to recycle them to improve the cost economics. For example,

the synthetic medium used in current work to generate BDO contained $(NH_4)_2SO_4$, therefore, the salt present in the bottom phase can be recycled for fermentative production of BDO. In a recent study, reverse osmosis, sugaring and salting out methods were employed to separate BDO accumulated by *Enterobacter cloacae* from glucose. The best results with 86% BDO recovery were obtained with salting out method using 20% (w/v) isopropanol and 20% (w/v) $(NH_4)_2SO_4$.[36] The recovery yield was enhanced to 99% using salt present in the bottom layer and solvent recycled with rotary evaporator. The results from this study demonstrate significant BDO titers from hemicellulosic hydrolysate along with promising BDO recovery. The process appears attractive from economic viewpoint and definitely shows a silver lining for xylose based industrial BDO production. To the best of our knowledge, this is the first report demonstrating BDO production using xylose rich hydrolysate from SCB along with recovery.

Conclusions

The efficient and rapid utilization of xylose is a prerequisite for sustainable and cost-effective production of BDO from LCB. The current study highlights the potential of *E. ludwigii* as a BDO producer from pure as well as SCB derived xylose and separation of accumulated BDO. Since the bacterium can efficiently ferment glucose and xylose to BDO with a high yield, it can be a promising strain for commercial BDO production from lignocellulosic feedstocks. To the best of our knowledge, there is no report: where, *E. ludwigii* has been used for the BDO production using pure/crude xylose as carbon source; using xylose-rich hydrolysate from SCB for biomanufacturing of BDO; with upstream and downstream of BDO together. Despite the encouraging results, more research work is required to improve the production and recovery parameters. *E. ludwigii* could be a good starting point for metabolic, evolutionary, and bioprocess engineering strategies to design novel hosts to meet commercial BDO manufacturing requirements. Further work in this direction using pure and crude xylose is underway along with optimization of product recovery process.

Supporting information

The metabolic pathway for BDO production from xylose and the by-product profile obtained during fed-batch fermentation of *E. ludwigii* using pure xylose, detoxified and non-detoxified xylose-rich SCB hydrolysates.

Acknowledgements

We are grateful to Ineuvo Ltd for funding this work. We are thankful to Cranfield University for providing facilities for conducting experiments. The funders had no role in study design, data collection, analysis, decision to publish, nor preparation of the article.

Contributions

YA and VN carried out all the experimental work and collected the data. VK analysed the data and wrote the manuscript. FC, DA, AKC, SM and AK were involved in proofreading the manuscript and revised the manuscript critically. All authors have read and approved the final manuscript.

Corresponding author

Correspondence to Vinod Kumar.

Consent for publication and Competing interests

The authors declare that they have no competing interests and give consent for publication.

Availability of Data and Materials

All data generated and analysed during this study are included in the manuscript.

References

(1) Marwoto, B.; Nakashimada, Y.; Kakizono, T.; Nishio, N. Enhancement of (R,R)-2,3-butanediol production from xylose by *Paenibacillus polymyxa* at elevated temperatures. *Biotechnol. Lett.* **2002,** 24 (2), 109-114. https://doi.org/10.1023/A:1013894403987

(2) Gírio, F.M.; Carvalheiro, F.; Duarte, L.C. Deconstruction of the hemicellulose fraction from lignocellulosic materials into smple sugars. In d-Xylitol; Silva S.S.; Chandel, A.K. Eds.; Springer, Berlin and Heidelberg, **2012**; pp 3–37. https://doi.org/10.1007/978-3-642-31887-0_1

(3) Kumar, V.; Binod, P.; Sindhu, R.; Gnansounou, E.; Ahluwalia, V. Bioconversion of Pentose Sugars to Value Added Chemicals and Fuels: Recent Trends, Challenges and Possibilities. *Bioresour. Technol.* **2018**, *269* (June), 443–451. https://doi.org/10.1016/j.biortech.2018.08.042.

(4) Kwak, S.; Jo, J. H.; Yun, E. J.; Jin, Y. S.; Seo, J. H. Production of Biofuels and Chemicals from Xylose Using Native and Engineered Yeast Strains. *Biotechnol. Adv.* **2019**, *37* (2), 271–283. https://doi.org/10.1016/j.biotechadv.2018.12.003.

(5) Pérez, J.; Muñoz-Dorado, J.; De La Rubia, T.; Martínez, J. Biodegradation and Biological Treatments of Cellulose, Hemicellulose and Lignin: An Overview. *Int. Microbiol.* **2002**, *5* (2), 53–63. https://doi.org/10.1007/s10123-002-0062-3.

(6) Wang, X. X.; Hu, H. Y.; Liu, D. H.; Song, Y. Q. The Implementation of High Fermentative 2,3-Butanediol Production from Xylose by Simultaneous Additions of Yeast Extract, Na2EDTA, and Acetic Acid. *N. Biotechnol.* **2016**, *33* (1), 16–22. https://doi.org/10.1016/j.nbt.2015.07.004.

(7) Ji, X.-J.; Huang, H.; Ouyang, P.-K. Microbial 2,3-Butanediol Production: A State-of-the-Art Review. *Biotechnol. Adv.* **2011**, *29* (3), 351–364. https://doi.org/https://doi.org/10.1016/j.biotechadv.2011.01.007.

(8) Köpke, M.; Mihalcea, C.; Liew, F.; Tizard, J. H.; Ali, M. S.; Conolly, J. J.; Al-Sinawi, B.; Simpson, S. D. 2,3-Butanediol Production by Acetogenic Bacteria, an Alternative Route to Chemical Synthesis, Using Industrial Waste Gas. *Appl. Environ. Microbiol.* **2011**, *77* (15), 5467 LP – 5475. https://doi.org/10.1128/AEM.00355-11.

(9) Vivek, N.; Hazeena, S. H.; Alphy, M. P.; Kumar, V.; Magdouli, S.; Sindhu, R.; Pandey, A.; Binod, P. Recent Advances in Microbial Biosynthesis of C3 – C5 Diols: Genetics and Process Engineering Approaches. *Bioresour. Technol.* **2021**, *322* (October 2020), 124527. https://doi.org/10.1016/j.biortech.2020.124527.

(10) Amraoui, Y.; Prabhu A. A.; Vivek, N.; Coulon, F.; Chandel, A. K.; Willoughby, N.; Jacob, S.; Koutinas, A.; Kumar, V. Enhanced 2,3-Butanediol production by mutant *Enterobacter ludwigii* using Brewers' spent grain hydrolysate: Process optimization for a pragmatic biorefinery loom. *Chem. Engine. Journal* **2021,** (June 2021)**,** 130851. https://doi.org/10.1016/j.cej.2021.130851.

(11) Nisiotou, A. A.; Rantsiou, K.; Iliopoulos, V.; Cocolin, L.; Nychas, G. J. E. Bacterial Species Associated with Sound and Botrytis-Infected Grapes from a Greek Vineyard. *Int. J. Food Microbiol.* **2011**, *145* (2–3), 432–436. https://doi.org/10.1016/j.ijfoodmicro.2011.01.017.

(12) Maina, S.; Stylianou, E.; Vogiatzi, E.; Vlysidis, A.; Mallouchos, A.; Nychas, G. J. E.; de Castro, A. M.; Dheskali, E.; Kookos, I. K.; Koutinas, A. Improvement on Bioprocess Economics for 2,3-Butanediol Production from Very High Polarity Cane Sugar via Optimisation of Bioreactor Operation. *Bioresour. Technol.* **2019**, *274* (October 2018), 343–352. https://doi.org/10.1016/j.biortech.2018.11.001.

(13) Sun, L.H., Jiang, B. and Xiu, Z.L. Aqueous two-phase extraction of 2, 3-butanediol from fermentation broths by isopropanol/ammonium sulfate system. *Biotechnology letters*, 31, 371-376. https://doi.org/10.1007/s10529-008-9874-3

(14) Ping, Y., Ling, H.Z., Song, G., Ge, J.P., 2013. Xylitol produciton from non-detoxified corncob hemicellulose acid hydrolysate by Candida tropicalis. Biochem. Eng. J. 75, 86-91. https://doi.org/10.1016/j.bej.2013.03.022

(15) Yang, T.; Rao, Z.; Zhang, X.; Lin, Q.; Xia, H.; Xu, Z.; Yang, S. Production of 2,3-Butanediol from Glucose by GRAS Microorganism Bacillus Amyloliquefaciens. *J. Basic Microbiol.* **2011**, *51* (6), 650–658. https://doi.org/10.1002/jobm.201100033.

(16) Bezerra, T.L., Ragauskas, A.J. A review of sugarcane bagasse for second generation bioethanol and biopower production. Biofuels Bioprod Biorefin. **2016,** 10:634–47. https://doi.org/10.1002/bbb.1662

(17) Konde, K. S.; Nagarajan, S.; Kumar, V.; Patil, S. V.; Ranade, V. V. Sugarcane Bagasse Based Biorefineries in India: Potential and Challenges. *Sustain. Energy Fuels* **2021**, *5* (1), 52–78. https://doi.org/10.1039/d0se01332c.

(18) Liakou, V.; Pateraki, C.; Palaiogeorgou, A. M.; Kopsahelis, N.; Machado de Castro, A.; Guimarães Freire, D. M.; Nychas, G. J. E.; Papanikolaou, S.; Koutinas, A. Valorisation of Fruit and Vegetable Waste from Open Markets for the Production of 2,3-Butanediol. *Food Bioprod. Process.* **2018**, *108*, 27–36. https://doi.org/10.1016/j.fbp.2017.10.004.

(19) Yu, E. K. C.; Saddler, J. N. Fed-Batch Approach to Production of 2,3-Butanediol by Klebsiella Pneumoniae Grown on High Substrate Concentrations. *Appl. Environ. Microbiol.* **1983**, *46* (3), 630–635. https://doi.org/10.1128/aem.46.3.630-635.1983.

(20) Celińska, E.; Grajek, W. Biotechnological Production of 2,3-Butanediol-Current State and Prospects. *Biotechnol. Adv.* **2009**, *27* (6), 715–725. https://doi.org/10.1016/j.biotechadv.2009.05.002.

(21) Psaki, O.; Maina, S.; Vlysidis, A.; Papanikolaou, S.; de Castro, A. M.; Freire, D. M. G.; Dheskali, E.; Kookos, I.; Koutinas, A. Optimisation of 2,3-Butanediol Production by Enterobacter Ludwigii Using Sugarcane Molasses. *Biochem. Eng. J.* **2019**, *152* (April). https://doi.org/10.1016/j.bej.2019.107370.

(22) Häßler, T.; Schieder, D.; Pfaller, R.; Faulstich, M.; Sieber, V. Enhanced Fed-Batch Fermentation of 2,3-Butanediol by Paenibacillus Polymyxa DSM 365. *Bioresour. Technol.* **2012**, *124*, 237–244. https://doi.org/10.1016/j.biortech.2012.08.047.

(23) Cho, S.; Kim, T.; Woo, H. M.; Lee, J.; Kim, Y. Enhanced 2 , 3-Butanediol Production by Optimizing Fermentation Conditions and Engineering Klebsiella Oxytoca M1 through Overexpression of Acetoin Reductase. **2015**, 1–16. https://doi.org/10.1371/journal.pone.0138109

(24) Biebl, H.; Zeng, A. P.; Menzel, K.; Deckwer, W. D. Fermentation of Glycerol to 1,3-Propanediol and 2,3-Butanediol by Klebsiella Pneumoniae. *Appl. Microbiol. Biotechnol.* **1998**, *50* (1), 24–29. https://doi.org/10.1007/s002530051251.

(25) Petrov, K.; Petrova, P. High Production of 2,3-Butanediol from Glycerol by Klebsiella Pneumoniae G31. *Appl. Microbiol. Biotechnol.* **2009**, *84* (4), 659–665. https://doi.org/10.1007/s00253-009-2004-x.

(26) Petrov, K.; Petrova, P. Enhanced Production of 2,3-Butanediol from Glycerol by Forced PH Fluctuations. *Appl. Microbiol. Biotechnol.* **2010**, *87* (3), 943–949. https://doi.org/10.1007/s00253-010-2545-z

(27) Wang, A.; Wang, Y.; Jiang, T.; Li, L.; Ma, C.; Xu, P. Production of 2,3-Butanediol from Corncob Molasses, a Waste by-Product in Xylitol Production. *Appl. Microbiol. Biotechnol.* **2010**, *87* (3), 965–970. https://doi.org/10.1007/s00253-010-2557-8.

(28) Trček, J.; Mira, N. P.; Jarboe, L. R. Adaptation and Tolerance of Bacteria against Acetic Acid. *Applied Microbiology and Biotechnology*. 2015, pp 6215–6229. https://doi.org/10.1007/s00253-015-6762-3.

(29) Kuenz, A.; Jäger, M.; Niemi, H.; Kallioinen, M.; Mänttäri, M.; Prüße, U. Conversion of Xylose from Birch Hemicellulose Hydrolysate to 2,3-Butanediol with Bacillus Vallismortis. *Fermentation* **2020**, *6* (3), 86. https://doi.org/10.3390/fermentation6030086.

(30) Cheng, K.-K.; Liu, Q.; Zhang, J.-A.; Li, J.-P.; Xu, J.-M.; Wang, G.-H. Improved 2,3-Butanediol Production from Corncob Acid Hydrolysate by Fed-Batch Fermentation Using Klebsiella Oxytoca. *Process Biochem.* **2010**, *45* (4), 613–616. https://doi.org/10.1016/J.PROCBIO.2009.12.009.

(31) Zhang, C.; Li, W.; Wang, D.; Guo, X.; Ma, L.; Xiao, D. Production of 2,3-Butanediol by Enterobacter Cloacae from Corncob-Derived Xylose. *Turkish J. Biol.* **2016**, *40* (4), 856–865. https://doi.org/10.3906/biy-1506-66.

(32) Xiao-Xiong, W.; Hong-Ying, H.; De-Hua, L.; Yuan-Quan, S. The implementation of high fermentative 2, 3-butanediol production from xylose by simultaneous additions of yeast extract, Na (2) EDTA, and acetic acid. *NEW BIOTECH.* **2016**., *33*(1), 16-22. https://doi.org/10.1016/j.nbt.2015.07.004

(33) Kim, S.J.; Hee, J.S.; Jin, W.K.; Ye, G.L.; Yong, C.P.; Jin, H.S. Enhanced production of 2, 3-butanediol from xylose by combinatorial engineering of xylose metabolic pathway and cofactor regeneration in pyruvate decarboxylase-deficient Saccharomyces cerevisiae. *Bioresource technology,* **2017**, 245, 1551-1557. https://doi.org/10.1016/j.biortech.2017.06.034.

(34) Xiu, Z. L.; Zeng, A. P. Present state and perspective of downstream processing of biologically produced 1, 3-propanediol and 2, 3-butanediol. *Appl. microbiol. and biotechnol,* **2008**., 78(6), 917-26. https://doi.org/10.1007/s00253-008-1387-4

(35) Xie, S.; Zhang, Y.; Zhou, Y.; Wang, Z.; Yi, C.; Qiu, X. Salting-out of bio-based 2, 3-butanediol from aqueous solutions. *J Chemical Technol. & Biotechnol.* **2017**, 92(1), 122-32. https://doi.org/10.1002/jctb.4999

(36) Priya, A.; Dureja, P.; Rathi, R.; Lal, B. Comparative assessment of separation techniques for downstream processing of 2, 3-Butanediol. *Fuel.* **2021,** 15, 292:120351.

https://doi.org/10.1016/j.fuel.2021.120351

(37) Jansen, N.; Flickinger, M. C.; Tsao, G. T. Production of 2,3-Butanediol from D-Xylose by Klebsiella oxytoca ATCC 8724. Biotechnol. and Bioeng. **1984**, *XXVI*, 362–369.https://doi.org/10.1002/bit.260260411.

(38) Grover, B. P.; Garg, S. K.; Verma, J. Production of 2,3-Butanediol from Wood Hydrolysate by Klebsiella Pneumoniae. *World J. Microbiol. Biotechnol.* **1990**, *6* (3), 328–332. https://doi.org/10.1007/BF01201306.

(39) Kim, S. J.; Seo, S. O.; Park, Y. C.; Jin, Y. S.; Seo, J. H. Production of 2,3-Butanediol from Xylose by Engineered Saccharomyces Cerevisiae. *J. Biotechnol.* **2014**, *192*, 376–382. https://doi.org/10.1016/j.jbiotec.2013.12.017.

(40) Guo, X. W.; Zhang, Y.; Li, L. L.; Guan, X. Y.; Guo, J.; Wu, D. G.; Chen, Y. F.; Xiao, D. G. Improved xylose tolerance and 2, 3-butanediol production of Klebsiella pneumoniae by directed evolution of rpoD and the mechanisms revealed by transcriptomics. Biotechnology for biofuels. 2018 Dec;11(1):1-8. https://doi.org/10.1186/s13068-018-1312-8

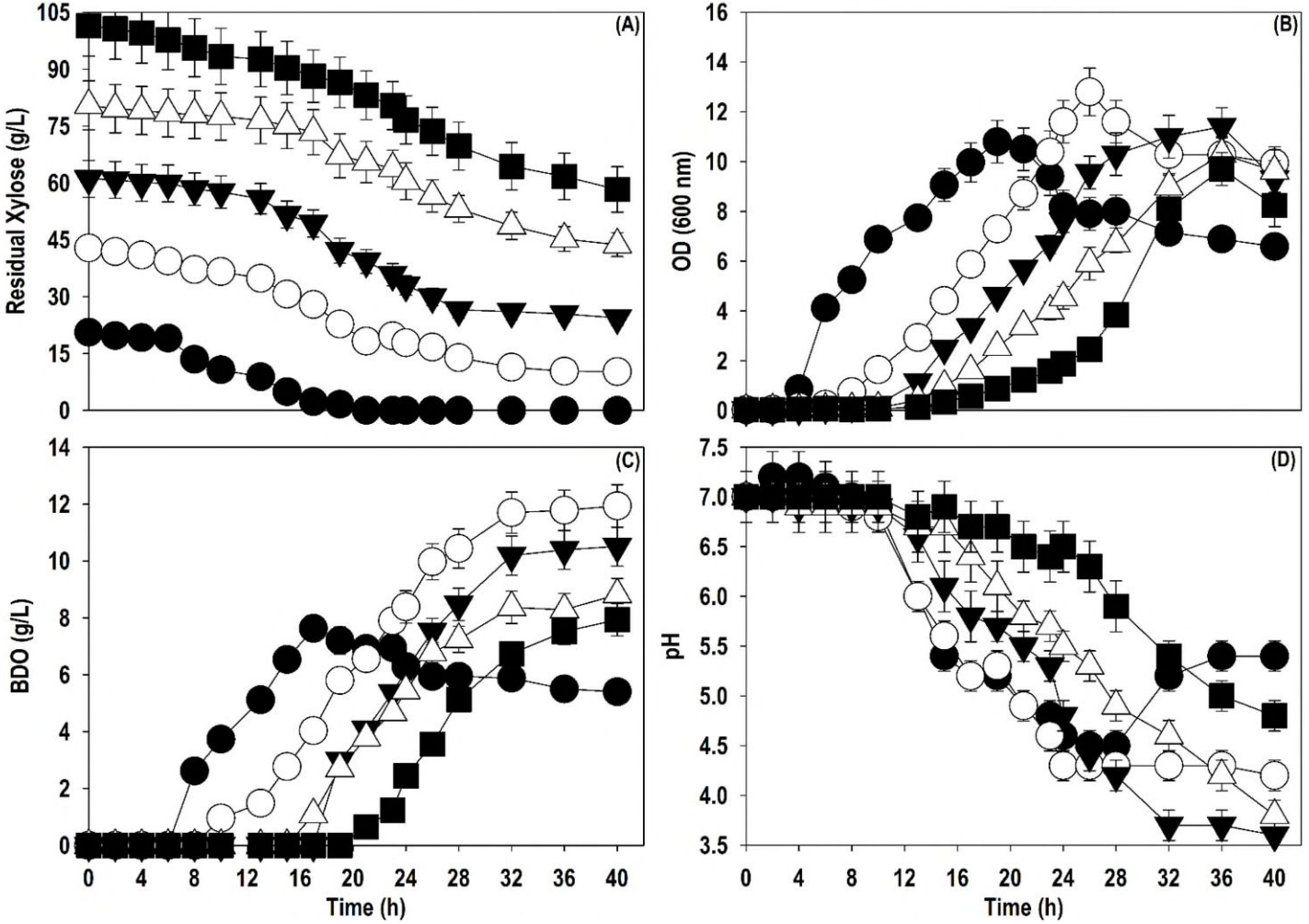


Figure 1: Shake flask cultivation of *E. ludwigii* at different initial levels of pure xylose: (A) xylose; (B) OD_{600}; (C) BDO; (D) pH. Symbols: filled circle (20 g/L), empty circle (40 g/L), filled triangle down (60 g/L), empty triangle (80 g/L) and filled square (100 g/L).

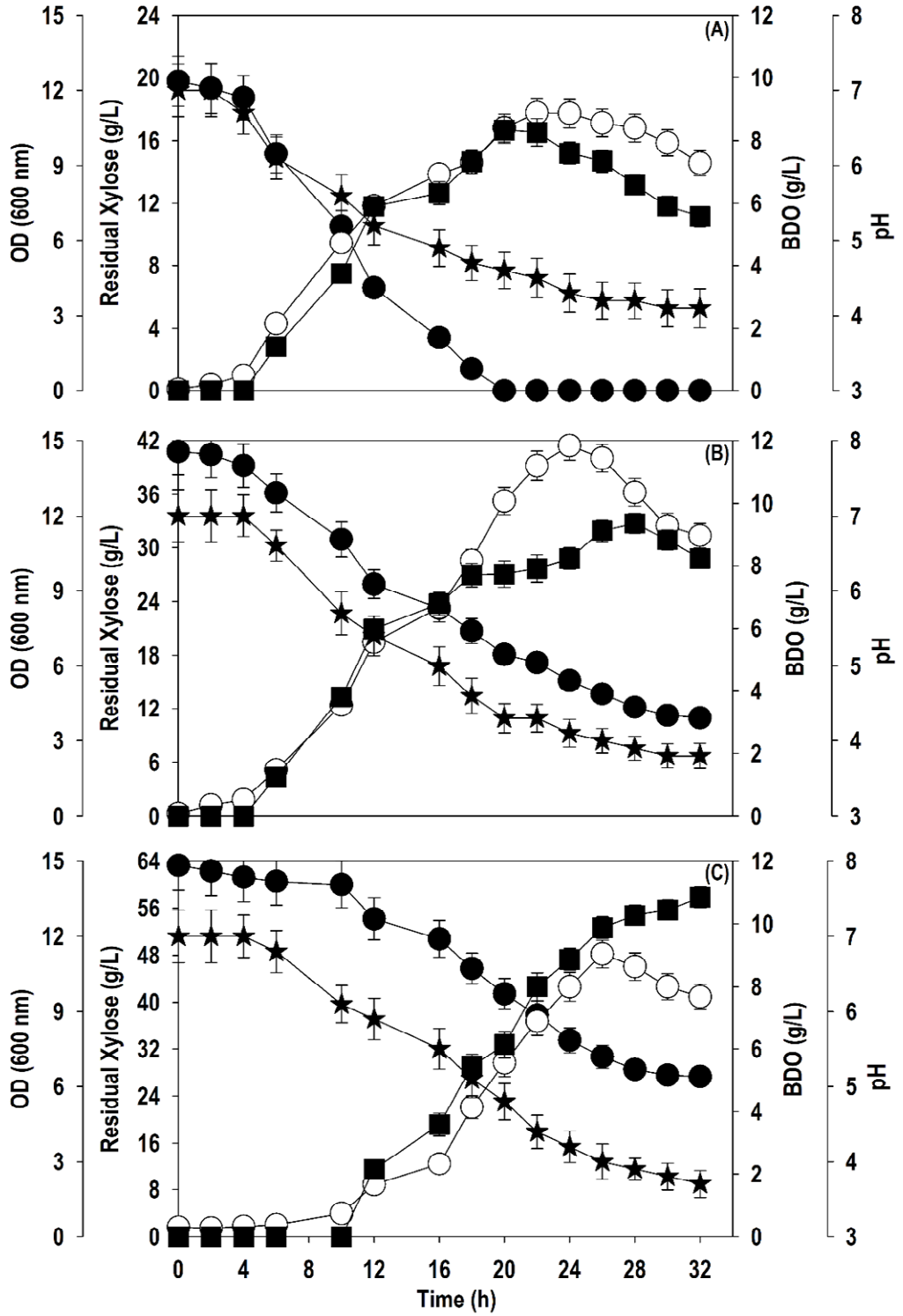


Figure 2: BDO fermentation in batch cultures by *E. ludwigii* using SCB-derived detoxified hemicellulosic hydrolysate at different xylose levels: (A) 20 g/L; (B) 40 g/L; (C) 60 g/L. Symbols: filled circle (xylose), empty circle (OD_{600}), filled square (BDO) and filled star (pH).

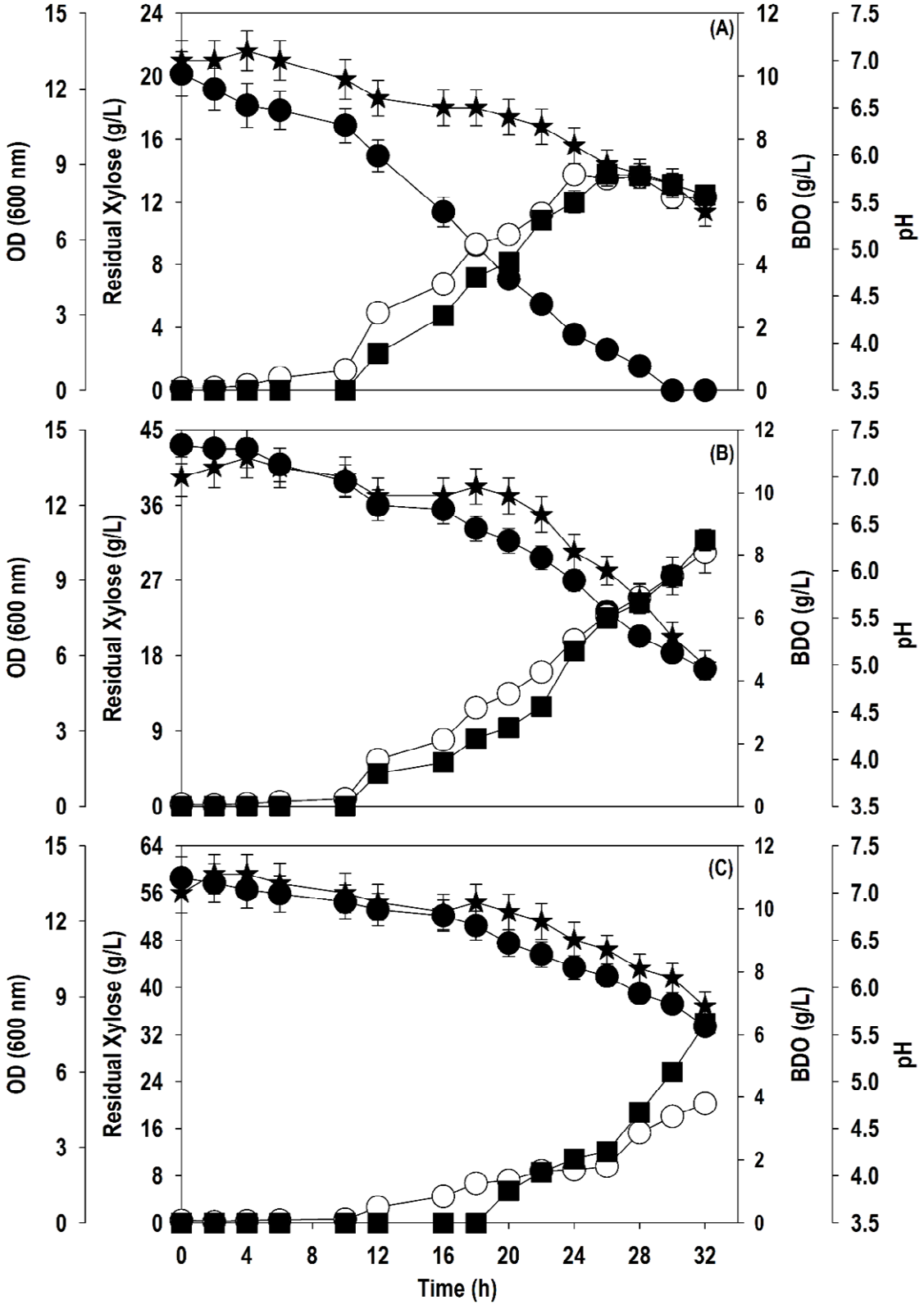


Figure 3: Kinetics of residual xylose, OD_{600}, pH and BDO production by *E. ludwigii* in shake flask at different initial xylose levels in SCB-derived non-detoxified hemicellulosic hydrolysate: (A) 20 g/L; (B) 40 g/L; (C) 60 g/L. Symbols: filled circle (xylose), empty circle (OD_{600}), filled square (BDO) and filled star (pH).

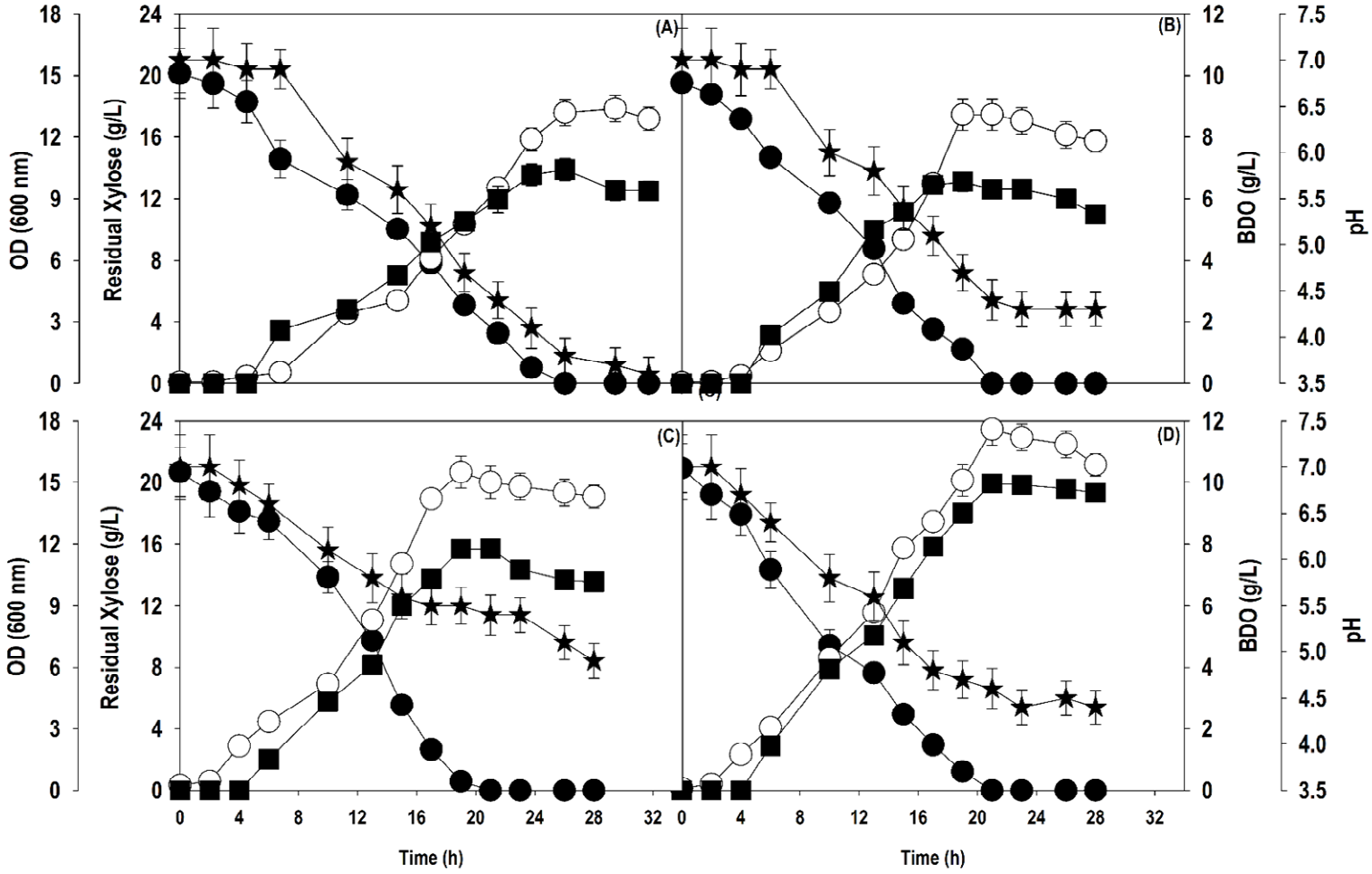


Figure 4: Effect of different nitrogen sources on xylose consumption, OD_{600}, BDO formation and pH from batch cultivations of *E. ludwigii* in shake flask: (A) control; (B) urea; (C) corn steep liquor; (D) yeast extract. Symbols: filled circle (xylose), empty circle (OD_{600}), filled square (BDO) and filled star (pH).

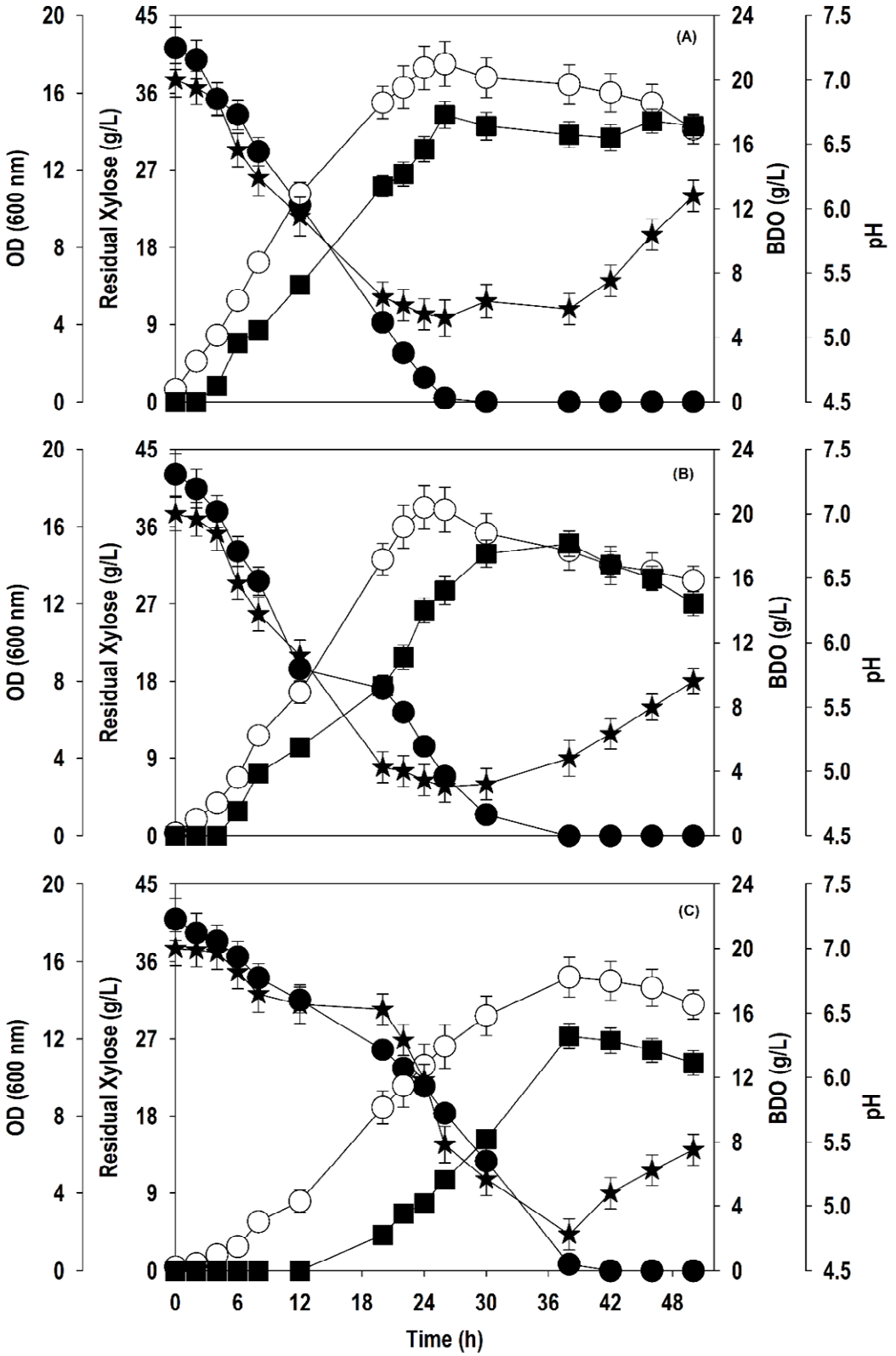


Figure 5: Variation in xylose consumption, OD_{600}, BDO formation and pH during batch cultivation of *E. ludwigii* in bioreactor on: (A) pure xylose; (B) detoxified SCB-derived xylose rich detoxified hydrolysate; (C) SCB-derived xylose rich non-detoxified hydrolysate. Symbols: filled circle (xylose), empty circle (OD_{600}), filled square (BDO) and filled star (pH).

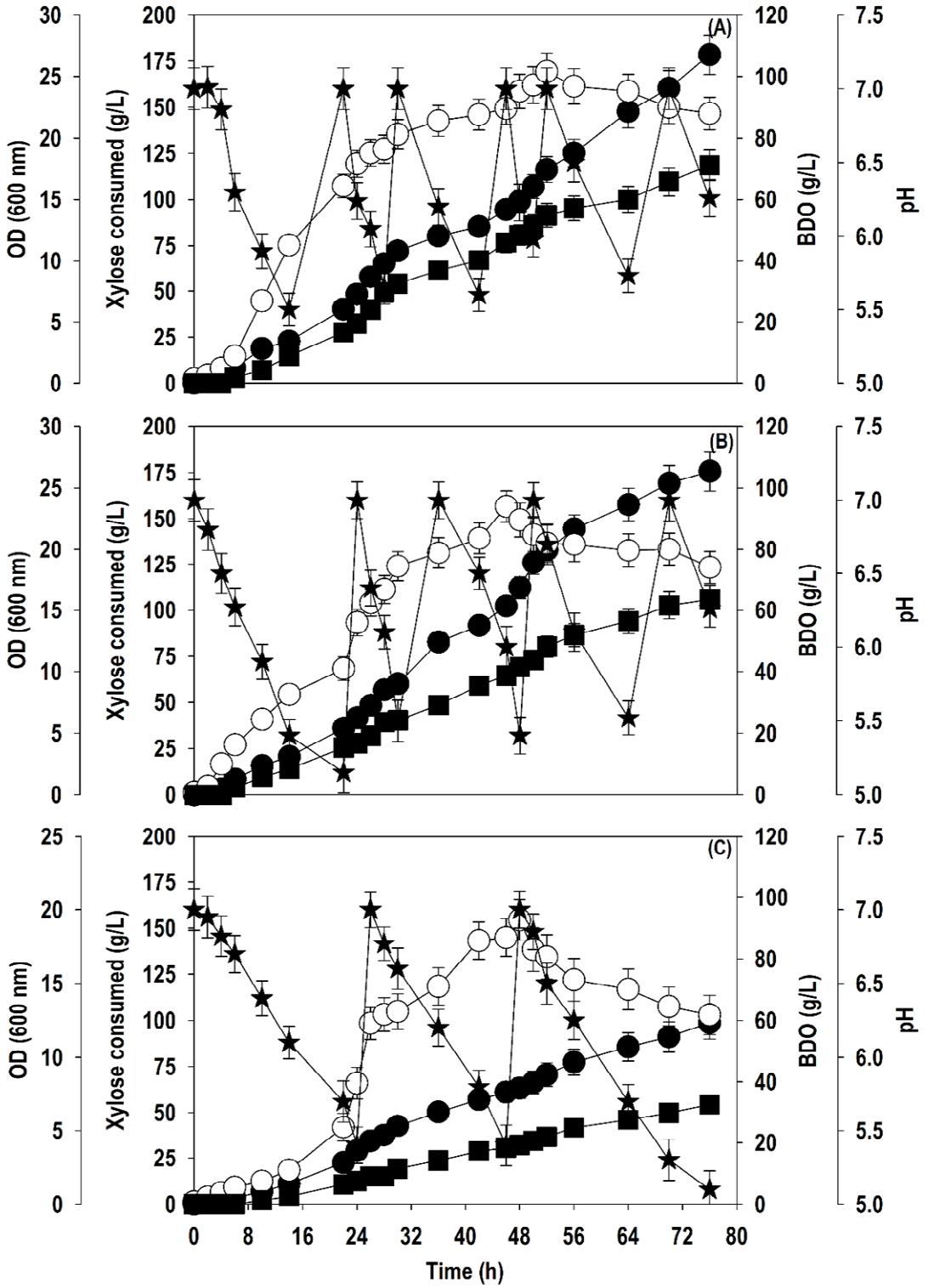


Figure 6: Time course profiles of xylose consumption, OD_{600}, BDO formation and pH during fed-batch cultivation of *E. ludwigii* in bioreactor on: (A) pure xylose; (B) SCB-derived xylose rich detoxified hydrolysate; (C) SCB-derived xylose rich non-detoxified hydrolysate. Symbols: filled circle (xylose), empty circle (OD_{600}), filled square (BDO) and filled star (pH).

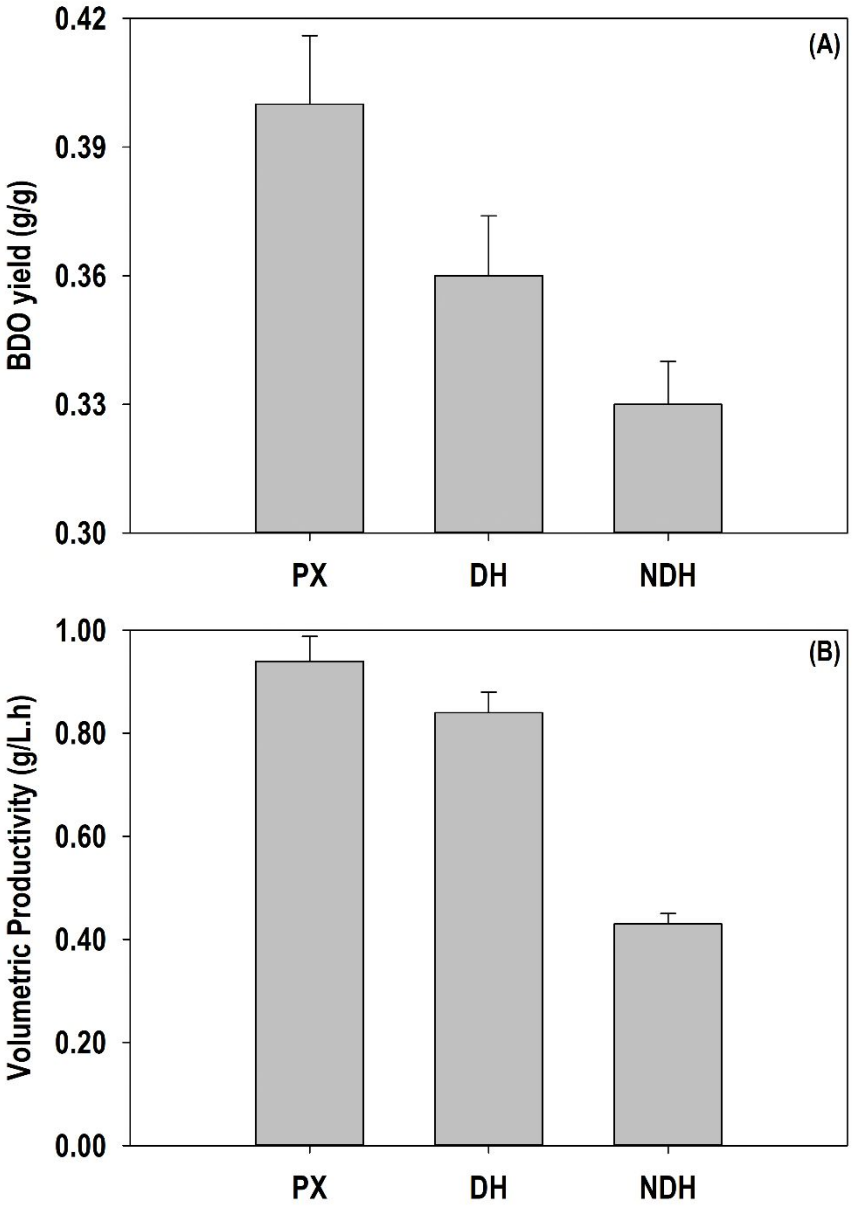


Figure 7: Comparison of yield and volumetric productivity of BDO accumulation from fed-batch cultivation of *E. ludwigii* in bioreactor. Abbreviations: PX – Pure xylose; DH – Detoxified hydrolysate; NDH – Non-detoxified hydrolysate.

Table 1: Salting out extraction of BDO from fermentation broth accumulated on pure xylose and detoxified xylose rich SCB hydrolysate.

Feedstock	**Partition coefficient**		**Recovery yield (%)**	
	K_{BDO}	**$K_{Acetoin}$**	**Y_{BDO}**	**$Y_{Acetoin}$**
Pure Xylose	7.33 ± 0.55	7.37 ± 0.58	87.99 ± 6.42	88.05 ± 6.54
Detoxified xylose rich SCB hydrolysate	6.62 ± 0.52	7.11 ± 0.58	86.87 ± 5.89	87.68 ± 6.32

Table 2: Summary of microbial BDO production from pure/crude xylose

Microorganism	Feedstock	Complex nitrogen source(s) (g/L)	Fermentation mode	pH	BDO			Reference
					Titer (g/L)	Yield (g/g)	Productivity (g/L. h)	
Paenibacillus polymyxa	Pure xylose	YE (5), T (5)	Batch	Uncontrolled	5.1	0.26	0.21	(1)
K. pneumoniae	Pure xylose	YE (35.2)	Fed-batch	Controlled	42.7	0.47	1.17	(32)
Bacillus vallismortis B-14891	Pure xylose	YE (10)	Batch	Controlled	26.5	0.32	1	(29)
B. vallismortis B-14891	Detoxified UF of birchwood hemicellulosic hydrolysate	YE (10)	Batch	Controlled	22.7	0.43	0.63	(29)
B. vallismortis B-14891	Detoxified NF of birchwood hemicellulosic hydrolysate	YE (10)	Batch	Controlled	15.3	0.40	0.46	(29)
K. oxytoca	Pure xylose	YE (1.5)	Batch	Controlled	30.3	0.52	1.26	(30)
K. oxytoca	Detoxified corncob acid hydrolysate	YE (1.5)	Fed-batch	Controlled	35.7	0.50	0.59	(30)
Enterobacter cloacae	Xylose solution	CL (20)	Fed-batch	Controlled	81.4	0.39	0.72	(31)
S.cerevisiae	Pure xylose	YE (10), BP (20)	Fed-batch	Uncontrolled	69.2	0.38	0.28	(33)
Klebsiella oxytoca	Pure xylose	None	Batch	Controlled	29.7	0.30	1.35	(37)
K. pneumoniae	Wood acid hydrolysate	Malt extract	Batch	Controlled	13.3	0.29	0.28	(38)
Saccharomyces cerevisiae	Pure xylose	YE (10), BP (20)	Batch	Uncontrolled	20.7	0.27	0.18	(39)
K. pneumoniae	Pure xylose	YE (10)	Batch	Controlled	38.6	-	0.62	(40)
Enterobacter ludwigii	Pure xylose	YE (2)	Fed-batch	Cyclic control	71.1	0.40	0.94	This study

E. ludwigii	Detoxified xylose rich SCB hydrolysate	YE (2)	Fed-batch	Cyclic control	63.5	0.36	0.84	This study
E. ludwigii	Non-detoxified xylose rich SCB hydrolysate	YE (2)	Fed-batch	Cyclic control	32.7	0.33	0.43	This study

BP – Bactopeptone; CL – Corn steep liquor powder; YE – Yeast extract; T – Tryptone, UF- Ultrafitrate, NF- Nanofiltrate